HIGH-PRESSURE LIQUID-SOLID CHROMATOGRAPHY OF THE ECDYSONES — INSECT MOLTING HORMONES

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ABSTRACT

A rapid, high-pressure liquid-solid chromatographic technique was developed for the separation, detection and analysis of ecdysteroids. The advantages in using this system for the identification of ecdysteroids in combination with NMR and mass spectrometry and its potential application in analyzing material from biological and synthetic systems are presented.

INTRODUCTION

Although the molting hormones (ecdysones) are present in small quantities in insects, considerable body of knowledge concerning their structure, metabolism, and biosynthetic precursors has been accumulated (1, 2, 3, 4, 5). The micro determination of these hormones has been carried out primarily through the use of a variety of biological assays (4). However, studies concerned with determinations such as the mode of action, specific metabolism, and excretion require that these hormones and their metabolites be isolated, separated, and identified. Such identifications have been accomplished by a combination of column chromatography, thin-layer chromatography

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(TLC), high-pressure liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and mass spectrometry. Also, efforts to improve techniques for detection, separation, identification, and microquantitation of ecdysteroids have resulted in the development of gas-liquid chromatographic analyses of trimethylsilyl ether derivatives of ecdysones (6, 7, 8). In addition, gas-phase microanalyses of trimethylsilyl ether and heptafluorobutyrate derivatives of zooecdysones and their quantitative estimation by mass spectrometry recently led to the detection of unidentified ecdysones in pupae of the silkworm, Bombyx mori (L.), at different developmental stages (9). High-pressure reverse-phase liquid-solid chromatography (HP-LSC) using an Amberlite XAD-2 column (10) has also been used successfully to separate many phytoecdysones (11) and insect ecdysones (12) though several hours were required for the analysis. Finally, the time required for HP-LSC analyses for certain ecdysones has been shortened by using reverse-phase chromatography on a "Poragel PN" column (13).

In the present paper we report the rapid separation by HP-LSC on Corasil II of three of the four known insect ecdysones, certain of their 5 α-isomers, and certain possible precursors of the ecdysones. Additional advantages in using this method in analyzing material from biological systems are also discussed.

METHODS AND MATERIALS

HP-LSC analyses were made at ambient temperature on a Dupont 830 liquid chromatograph equipped with a refractometer and a UV detector with a set wavelength of 254 nm. Only the UV detector was

used in these analyses. Three stainless steel U-shaped columns, 1.0 m X 2.0 mm ID, were dry packed with Corasil II (37-50μ) (Waters Associates, Milford, MA. 01757) with tapping and suction and were connected in series. The solvent system consisted of various mixtures of chloroform and 95% ethanol. The concentrations of 95% ethanol in chloroform, the operating pressures, and the solvent flow rates are given in the figure legends. Double filtered house air was used to pressurize the system. The solvent reservoir was not perfused with nitrogen though the solvent was degassed by applying vacuum to the reservoir. The compounds were injected in 5μl of methanol and the peaks (recorded at 0.08 Absorbance Units Full Scale) represent approximately 2.5μg of each compound (Figure 2).

Compounds 1, 2, 3, 5, and 7 were synthesized in our laboratory. Compound 6 (α-ecdysone) was a gift from Dr. P. Hocks, Schering Laboratory, Berlin, Germany. Compounds 4, 8, and 9 were obtained from larval frass (14), pupae (15), and embryos (16) of the tobacco hornworm, Manduca sexta (L.), respectively. All compounds exceeded 95% purity as indicated by HP-LSC, TLC, and UV analyses.

RESULTS AND DISCUSSION

Although we are mainly concerned in this paper with the HP-LSC of ecdysteroids of high purity, the technique has also been used successfully in our metabolic studies. Thus, we will discuss briefly the techniques used and the solutions found for problems encountered during the actual analyses of ecdysones obtained from biological systems.

Crude extracts from biological systems are purified by partitioning with a specific solvent system, column chromatographed on Unisil (100 - 200 mesh, Clarkson Chemical Co., Williamsport, PA.), and then further fractionated by column chromatography into apolar, polar, and more polar fractions (14). The material for HP-LSC analysis is dissolved into a measured quantity of methanol, and all insoluble material is separated by centrifugation or filtration. Only the clear

Key to Compounds in Figures 1, 2, and 3.

1 = 2β,14α-Dihydroxy-5β-cholest-7-en-3,6-dione

2 = 2β,3β,14α-Trihydroxy-5α-cholest-7-en-6-one

3 = 2β,3β,14α-Trihydroxy-5β-cholest-7-en-6-one

4 = 22-Deoxyecdysone = 2β,3β,14α,25-Tetrahydroxy-5β-cholest-7-en-6-one

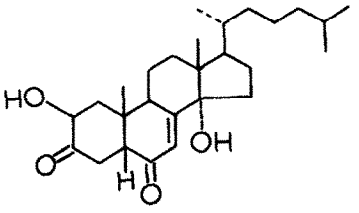
5 = 5α-α-Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5α-cholest-7-en-6-one

6 = α-Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

7 = 5α-20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5α-cholest-7-en-6-one

8 = 20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

9 = 26-Hydroxyecdysone = 2β,3β,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one



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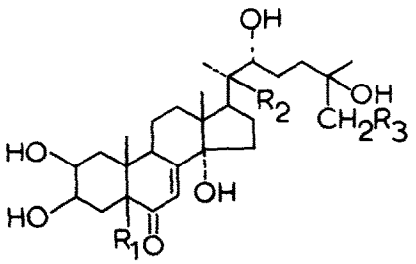
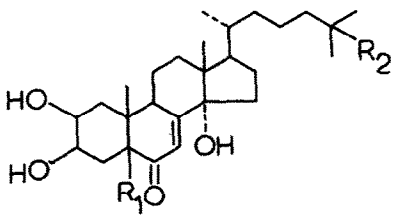


	R_1	R_2	R_3
5	α-H	H	H
6	β-H	H	H
7	α-H	OH	H
8	β-H	OH	H
9	β-H	H	OH



	R_1	R_2
2	α-H	H
3	β-H	H
4	β-H	OH

Fig. 1. Ecdysteroids used in this study.

supernatant is injected into the HP-LSC column since any part of an injection that remains on the column may change the chromatographic properties of the system.

Appropriate clean-up procedures minimize this effect. The analysis of extracts eventually leads to changes in column performance such as alterations in flow rate and symmetry of peaks and reduction in detector response. However, we have used the same column packing material for several months and have been able to regain original performance simply by passing methanol, and then chloroform through the column followed by the desired solvent mixture of chloroform–ethanol. A reduced flow rate can usually be corrected by cleaning the face of the inlet frit of the lead column. Because of this potential variability, appropriate standards should be injected either alone or in combination with a sample of the mixture being analyzed. Accordingly, HP-LSC retention times alone are not valid criteria for positive identification, and other methods of analyses such as TLC, NMR, and mass spectrometry should also be employed.

Interestingly, as with thin-layer chromatography, chloroform–ethanol proved to be the best solvent system for separating the ecdysones. Although the ultraviolet cut-off for chloroform is 245 nm and the ecdysones absorb in the UV at 244 nm, determination of microgram levels of these compounds was still possible without having to utilize maximum detector sensitivity.

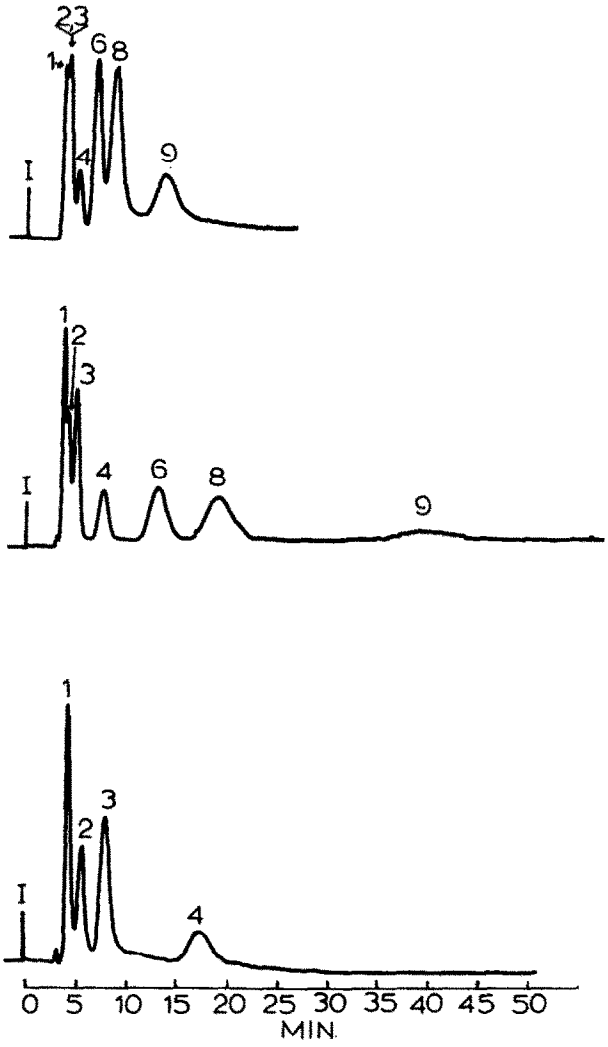


Fig. 2. Liquid-solid chromatography of ecdysones 1-4 and 6, 8, and 9. Top trace: 4:1 chloroform-ethanol; middle trace: 9:1 chloroform-ethanol; bottom trace: 14:1 chloroform-ethanol. Flow rate 1.5 ml/min. Pressure 1500 psi. I = injection.

In Figure 2, the top trace shows a rapid analysis by HP-LSC of ecdysteroids that contain from two to six hydroxyl groups. Although six of the seven compounds show separation in the top trace, four of the most polar compounds exhibited baseline separation when the solvent was changed from a 4:1 to a 9:1 chloroform-ethanol system. In the

bottom trace of Figure 2, the apolar steroids 1, 2, 3, and 4 were better separated by further decreasing the concentration of ethanol in the chloroform. Thus, in an actual analysis of ecdysteroids from a biological system, the 4:1 chloroform-ethanol solvent would be most ideal for a rapid analysis, especially where very polar products are expected. The unresolved apolar steroids could then be collected and rechromatographed for further separation and identification by using the less polar 14:1 chloroform-ethanol solvent system. However, if a gradient elution system of chloroform with increasing amounts of ethanol were used in the initial analysis, a baseline separation of these ecdysteroids could probably be obtained.

Our general procedure for processing the crude ecdysones, whether isolated from an *in vivo* or *in vitro* system, includes as a first step partial purification by column chromatography and the material is then further separated into apolar, polar, and more polar fractions (14). Thus, for HP-LSC analyses, our solvent system of choice would depend on which fraction is to be analyzed.

Figure 3 shows the separation of α-ecdysone and its 5α-isomer and 20-hydroxyecdysone and its 5α-isomer. Even though α-ecdysone and the 5 α-isomer of 20-hydroxyecdysone cochromatograph in the solvent systems of the upper and middle traces, the two ecdysteroids show distinguishable peaks in a less polar system (bottom trace). Either compound could be completely separated from the other by collecting portions of the peaks eluted in the 14:1 chloroform-ethanol solvent system. Subsequent

recycling of these peaks would most likely result in baseline separation.

In the middle trace of Figure 3, the peak containing compounds 6 and 7 does not discernibly indicate a mixture. However, the collection and subsequent mass and NMR spectral analyses of this material would have readily indicated a mixture. By utilizing this method of analysis we have routinely determined whether isolated material appearing in the LS chromatogram is an ecdysone.

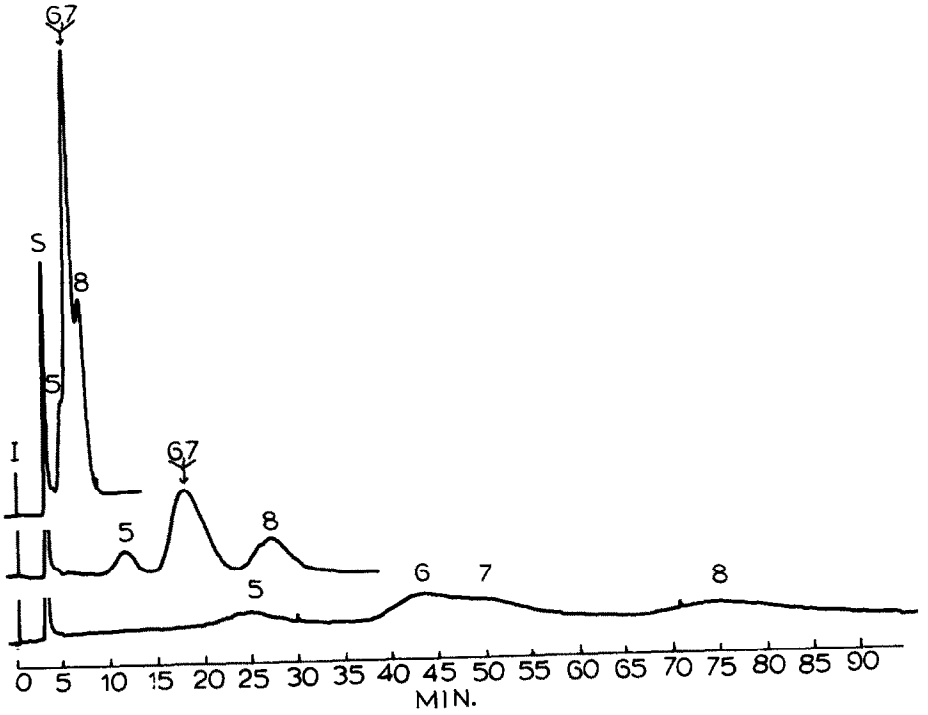


Fig. 3. Liquid-solid chromatography of ecdysones 5-8. Top trace: 4:1 chloroform-ethanol; middle trace: 9:1 chloroform-ethanol; bottom trace: 14:1 chloroform-ethanol. Flow rate 1.63 ml/min. Pressure: 1500 psi. I = injection. S = solvent plus apolar impurity.

The combination of HP-LSC and mass spectrometry has permitted us to examine non-radiolabeled ecdysone metabolites at microgram levels. We are also able to develop and determine the appropriate conditions for *in vitro* systems that would produce the best possible yields of certain metabolites and to collect and accumulate material for structural characterization.

Our Corasil II HP-LSC method clearly enhances the value of HP-LSC as an analytical method for analyses of ecdysone mixtures. The availability of adsorbents with a higher capacity and efficiency than Corasil II should further augment the utility of HP-LSC for the detection and analysis of new ecdysones of plants and insects as well as for exploratory investigations of natural and synthetic products.

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